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Supplemental information

**Post-weaning A1/A2 β -casein milk intake
modulates depressive-like behavior, brain
 μ -opioid receptors, and the metabolome of rats**

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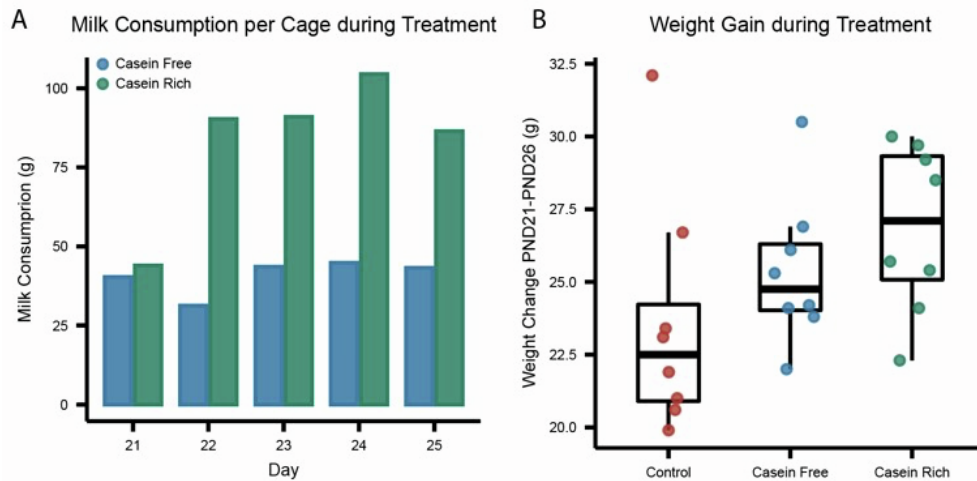


Figure S1. Milk consumption and weight gain during intervention in Casein Study, related to Figure 1

A) Animals consumed more casein-rich milk compared to casein-free milk per cage. B) Higher milk consumption did not result in significant differences in weight gain between groups during treatment. ANOVA followed by Tukey's HSD post-hoc test.

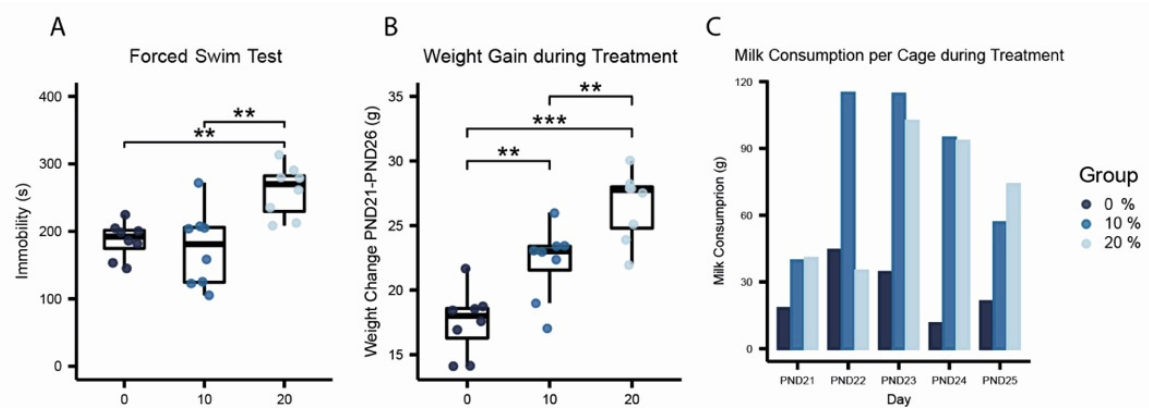


Figure S2. Concentration dependent effect of milk casein on milk consumption, body weight and stress-induced immobility behaviour, related to Figure 1

Casein-free milk formula (Special Diet services, Cambridge, UK) (0% casein) was spiked with 10% or 20% w/v casein (Sigma, Poole, UK) and provided to Male Wistar Albino rat pups upon weaning on PND21 until behavioural testing on PND25. A) Increased immobility time in 20% spiked milk group compared to both control and 10% spiked milk groups. No significant difference observed between control and 10% spiked milk group. B) Animals fed with 10% and 20% spiked milk gained significantly more weight compared to control. C) Animals appeared to consume per cage more spiked milk (both 10% w/w and 20% w/w) compared to casein-free milk. ANOVA followed by Tukey's HSD post-hoc test, significant p values are represented as ** $p < 0.01$, *** $p < 0.001$.

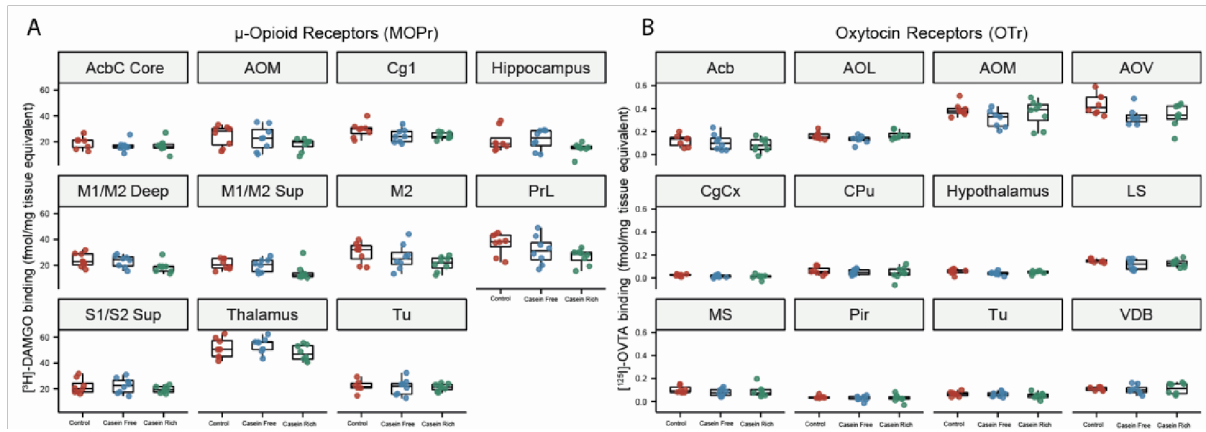


Figure S3. Brain autoradiography of mu-opioid and oxytocin receptors in Casein Study, related to Figure 1

Boxplots show first (lower) quartile, median and third (upper) quartile of A) mu-opioid receptors (MOPr) and B) oxytocin receptors (OTr). No significant differences were observed between groups (ANOVA followed by Tukey's HSD post-hoc test). AcbC, nucleus accumbens; AOM, anterior olfactory nucleus-medial; AOL, anterior olfactory nucleus-lateral; AOV, anterior olfactory nucleus-ventral; M1/M2 deep + supp, motor cortex deep or superficial respectively; M2, motor cortex; PrL, Prelimbic cortex; S1/S2 sup, somatosensory cortex superficial; Tu, olfactory tubercle; CgCx, cingulate cortex; CPu, caudateputamen; LS, lateral septum; MS, medial septum; Pir, piriform cortex; TU, Olfactory tubercle; VDB, vertical limb of the diagonal band Broca.

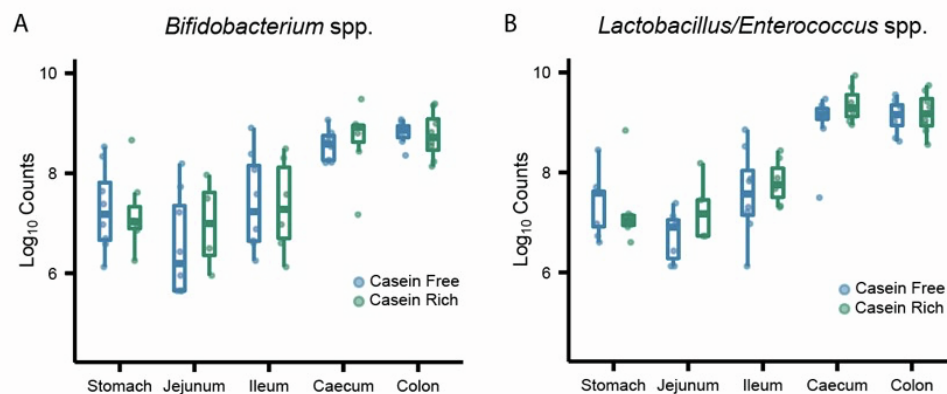


Figure S4. Fluorescence *in-situ* hybridisation (FISH) analysis of gut contents collected from animals in the Casein Study, related to Figure 1

No significant differences were observed between treatments for *Bifidobacteria* and *Lactobacilli/Enterococci* populations in any of the regions analysed (Wilcoxon test followed by Benjamini-Hochberg correction).

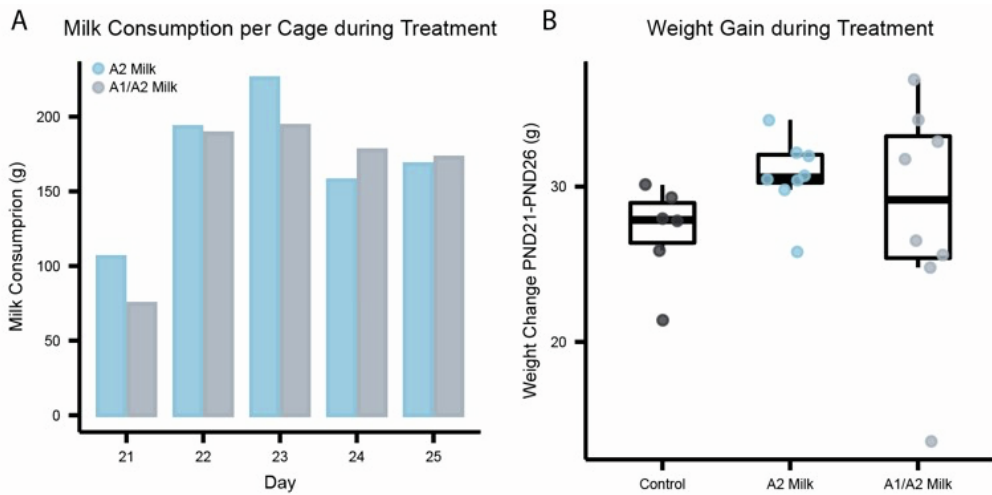


Figure S5. Milk consumption and weight gain during intervention in A1/A2 Study, related to Figure 3

A) No significant difference in milk consumption per cage was observed. B) No significant differences in weight gain between groups during treatment. ANOVA followed by Tukey's HSD post-hoc test.

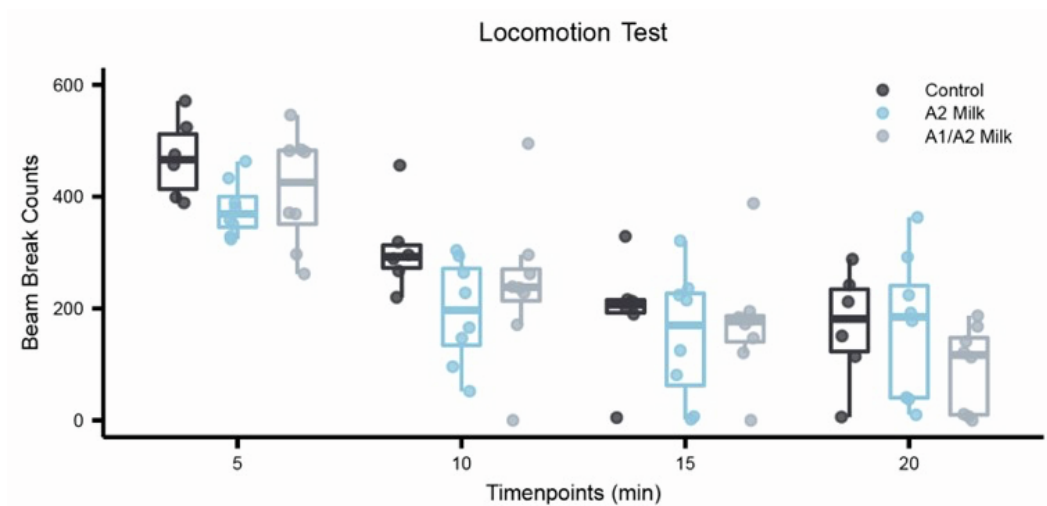


Figure S6. Locomotor activity test in A1/A2 Study, related to Figure 3

No significant differences were observed between the three groups at any of the timepoints studied. ANOVA followed by Tukey's HSD post-hoc test.

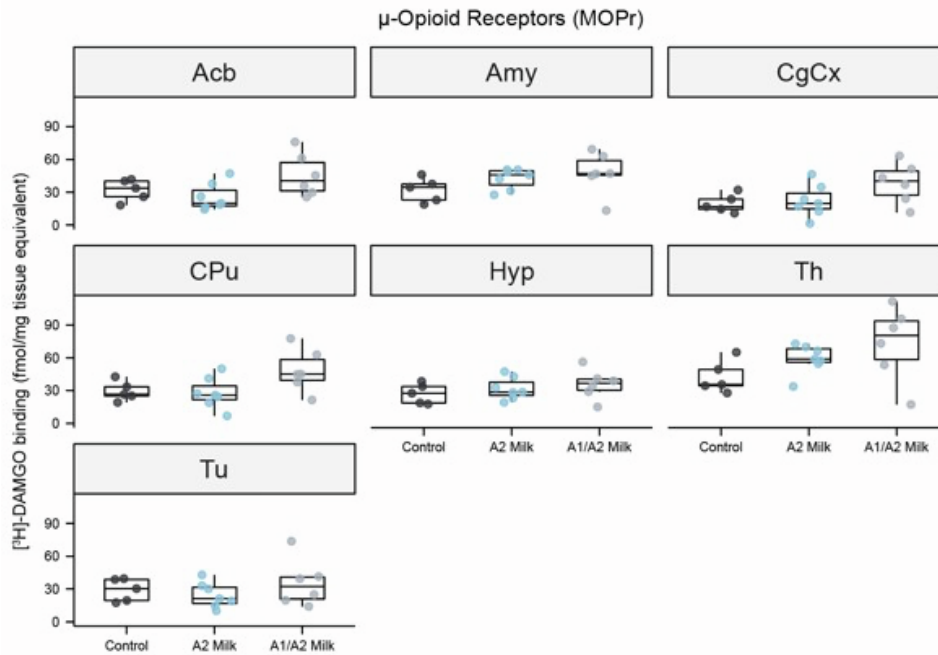


Figure S7. Brain autoradiography of mu-opioid receptors in A1/A2 Study, related to Figure 3
 No significant differences were observed in any of the regions measured between the study groups (ANOVA followed by Tukey's HSD post-hoc test). Boxplots show first (lower) quartile, median and third (upper) quartile. AcbC, nucleus accumbens; Amy, amygdala; CgCx, cingulate cortex; CPu, caudate-putamen; Hyp, hypothalamus; Th, thalamus; TU, olfactory tubercle.

Casein Rich Study - Rodent Chow Formula (B&K Universal Ltd)

	Total Diet (w/w)	Units
Crude Oil	2.5	%
Crude Protein *no casein	18	%
Moisture, Crude Fiber, Carbohydrate, Nitrogen Free Extracts (NEF) and Other	74	%
Ash	5.5	%
Ingredients	Wheat meal, barley meal, soya meal, wheat feed, fish meal, fats and oils, minerals and trace elements, molasses, amino acids, vitamins, micro minerals	

A1/A2 Milk Study - Rodent Chow Formula (SDS, Kent UK)

	Total Diet (w/w)	Units
Crude Oil	2.71	%
Crude Protein *no casein	14.38	%
Moisture, Crude Fiber, Carbohydrate, Nitrogen Free Extracts (NEF) and Other	76.91	%
Ash	6	%
Ingredients	Wheat, barley, wheat feed, de-hulled extracted toasted, soya, soya protein concentrate, macro minerals, soya oil, whey powder, amino acids, vitamins, micro minerals	

Table S1. Rodent Chow formulation, related to Figure 1 and Figure 3

Table depicting the composition of the rodent chow diets provided during the Casein Rich Study and A1/A2 Milk Study.

Casein Rich Milk Formula

	Results Fresh Wight Milk (Liquid)	Units
Moisture	80.67	%
Fat	3.78	%
Protein	5.3	%
	(4.24 Casein, 1.06 Whey)	
Fibre	0.18	%
Ash Raw	1.24	%
NFE (Nitrogen Free Extract)	8.83	%
Ingredients	Spray dried skimmed milk, vegetable and fats mix, spray dried milk albumin, spray dried whey products, vitamins and minerals mix	

A1/A2 Milk Formula

	Results Fresh Wight Milk (Liquid)	Units
Moisture	87.5	%
Fat	3.57	%
Protein	3.19	%
	(2.55 Casein, 0.64 Whey)	
Fibre	0	%
Ash Raw	0.7	%
NFE (Nitrogen Free Extract)	5.04	%

Casein Free Milk Formula

	Results Fresh Wight Milk (Liquid)	Units
Moisture	80.67	%
Fat	3.78	%
Protein	5.3	%
	(4.24 Soy, 1.06 Whey)	
Fibre	0.18	%
Ash Raw	1.24	%
NFE (Nitrogen Free Extract)	8.83	%
Ingredients	Whey powder, soy protein concentrate, maze oil, soya oil, vitamins and minerals mix	

A2 Milk Formula

	Results Fresh Wight Milk (Liquid)	Units
Moisture	87.6	%
Fat	3.62	%
Protein	3.25	%
	(2.6 Casein, 0.65 Whey)	
Fibre	0	%
Ash Raw	0.7	%
NFE (Nitrogen Free Extract)	4.83	%

Table S2. Milk formula composition of the utilized milks, related to Figure 1 and Figure 3

Table depicting the composition of each of the milk diets used. Analysis of milk was carried out by Special Diet services, Cambridge UK. % casein and whey composition was calculated based on 80%/20% protein ratio in bovine milk